Volume 213, number 1, 164–168 FEB 04489 March 1987

Demonstration of an epidermal growth factor-dependent 58 kDa phosphoprotein secreted by rat kidney fibroblasts

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Received 22 December 1986

Epidermal growth factor and 12-*O*-tetradecanoylphorbol-13-acetate increased the amount of $^{32}P_i$ found as phosphoserine in a major, hitherto not described 58 kDa phosphoprotein (pp58) secreted by normal rat kidney fibroblasts. Platelet-derived growth factor, insulin, nerve growth factor and fibroblast growth factor did not affect pp58 while transforming growth factor β decreased the accumulation of radioactivity into pp58. Cycloheximide, actinomycin D and ammonium chloride suppressed the labelling of pp58.

Epidermal growth factor; Phosphoprotein secretion; (Normal rat kidney fibroblast)

1. INTRODUCTION

Modification of cellular proteins by phosphorylation/dephosphorylation is assumed to play a key role in the mediation of biological effects elicited by peptide growth factors such as EGF, PDGF, NGF or insulin [1].

In extension of the view that the mediators of mitogenic signals are intracellular peptides, secreted polypeptides were proposed to play a messenger role outside the cell [2]. They could regulate the growth, attachment, directional migration or entry of cells into the S phase of the cell cycle [2]. Among them, phosphoproteins are possible candidates [3–5]. For example, a relationship has been demonstrated between cellular growth and the secretion of so called 'transformation-specific phosphoproteins' [4] or the phosphorylation level of fibronectin [5].

Therefore, to look for growth factor-dependent secreted phosphoproteins is of interest.

Here, we describe an as yet unrecognized EGF-dependent 58 kDa phosphoprotein secreted by normal rat kidney fibroblasts.

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Abbreviations: NRK, normal rat kidney fibroblasts (clone 49F); EGF, epidermal growth factor; PDGF, platelet-derived growth factor; FGF, fibroblast growth factor; TGF-β, transforming growth factor-β; TPA, 12-*O*-tetradecanoylphorbol-13-acetate

2. MATERIALS AND METHODS

Mouse EGF was isolated as described in [6]. Porcine insulin free of proinsulin was from Berlin-Chemie, GDR. The other growth factors were kindly provided by Drs C.-H. Heldin, Uppsala (PDGF), W. Birchmeier, Tübingen (TGF-β from human platelets) and E. Severin, Moscow (NGF). They were added to the cells with 0.01% bovine serum albumin (Serva). TPA used with 0.001% (v/v) acetone was from Sigma (USA).

Normal rat kidney fibroblasts (NRK cells), clone 49 F [7], were obtained from Dr B. Westermark, Uppsala. They were maintained in Eagle's minimal essential medium (Sifin, Berlin) containing 10%

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00145793/87/$3.50

calf serum. Cells were passaged weekly before reaching confluency.

For labelling experiments, confluent cells (10^6 cells) were obtained and the monolayers received fresh medium supplemented with 10% calf serum overnight. Next day the monolayers were washed three times with phosphate-free and serum-free medium supplemented with 20 mM Hepes, pH 7.3. Cells were then incubated in 1 ml of the same medium containing 0.1–0.5 mCi $^{32}P_i$ (spec. act. 5000 Ci/mmol; Central Institute of Nuclear Research, Dresden) in the presence or absence of different growth factors at 37°C for 3 h. The culture fluid was removed and centrifuged first at 3000 × *g* for 10 min, and then at 12000 × *g* for 1 min. For SDS-PAGE, 0.8 ml of the supernatant were precipitated at 4°C overnight by addition of 0.65 ml of 20% trichloroacetic acid/0.1 M P_i and haemoglobin at a final concentration of 0.1 mg/ml. The precipitate was washed with ethanol/ether (1:1), solubilized in 50 μl Laemmli sample buffer with 5% β-mercaptoethanol [8] and boiled for 3 min. Volumes of solubilized precipitates corresponding to equal incorporation of radioactivity into the cells (total acid-precipitable cpm) were applied to the gel. To ensure this, monolayers were treated with 10% trichloroacetic acid/0.1 M P_i at 4°C overnight, washed once with the same solution and dissolved in 1 N NaOH. Aliquots were taken for measurement of radioactivity and protein concentration [9]. Zero time incorporation was negligible. Stimulation of incorporation of ^{32}P into the cells by EGF was about 1.7-fold in comparison with the control cells.

SDS-PAGE was carried out according to Laemmli [8] using 7.5–15% linear gradient gels. Fixed and stained gels were dried and exposed to ORWO HS 11 film at −70°C with intensifying screens. Cytochrome *c* (12 kDa), soybean trypsin inhibitor (22 kDa), aldolase (40 kDa), ovalbumin (45 kDa) and bovine serum albumin (67 kDa) served as molecular mass standards. For phosphoamino acid analysis, secreted proteins were acid-precipitated as described above, washed four times with 10% trichloroacetic acid/0.1 M phosphate, twice with ethanol/ether (1:1) and hydrolysed in 6 N HCl for 1 h at 110°C in the presence of the phosphoamino acid standards. Electrophoresis of the hydrolysis products was carried out on thin-layer cellulose plates at pH 3.5 as described in [10].

3. RESULTS AND DISCUSSION

The exposure of NRK cells to EGF during a 3 h incubation with $^{32}P_i$ caused a 3–8-fold increase in the radioactivity incorporated into a major secreted phosphoprotein with a molecular mass of 58 kDa, designated pp58 (fig.1). Scanning the dried gel revealed that pp58 incorporated about 60–70% of the total radioactivity in the gel. The remaining activity was found to be associated with polypeptides migrating close to the main peak of radioactivity (see scan in fig.1). PDGF (lane 6), NGF (lane 7), insulin (lane 8), or FGF (not shown) did not exhibit any influence on the pattern of secreted phosphoproteins. EGF [11], insulin [11] or PDGF [12] all at concentrations we have used in the phosphorylation experiments were characterized as mitogens for NRK cells. However, EGF was the only growth factor which stimulated the labelling of pp58. TFD-β, which has been shown to inhibit the proliferation of NRK cells and to stimulate the secretion of fibronectin [13,14], reduced the accumulation of ^{32}P in pp58 below the level of control cells (fig.1b, lane 1). On the other hand, the phorbol ester TPA, similarly to EGF, caused a strong increase of ^{32}P incorporation into pp58 (fig.1, lane 9). This compound has been shown to bind directly to and activate protein kinase C [15].

In unfractionated cell homogenates pp58 was not observed (not shown), suggesting that pp58 is not a major cellular phosphoprotein.

As shown in fig.1, lane 3, the presence of cycloheximide during the 3 h labelling period was sufficient to abolish the appearance of pp58. This means that protein synthesis either of pp58 itself or of a modifying enzyme is necessary. In addition, the inhibitory effect of a low concentration of actinomycin D (lane 5) indicates the involvement of new transcription.

Further, ammonium chloride was tested in order to see whether acidic intracellular compartments are involved in phosphorylation, secretion or rapid degradation of pp58. Incorporation of ^{32}P into pp58 was completely prevented when cells were treated for 3 h with NH_4Cl in the presence of EGF (fig.1, lane 4). This finding strongly indicates that pp58 is not a lysosomal protein because these are known to be secreted in the presence of NH_4Cl [16].

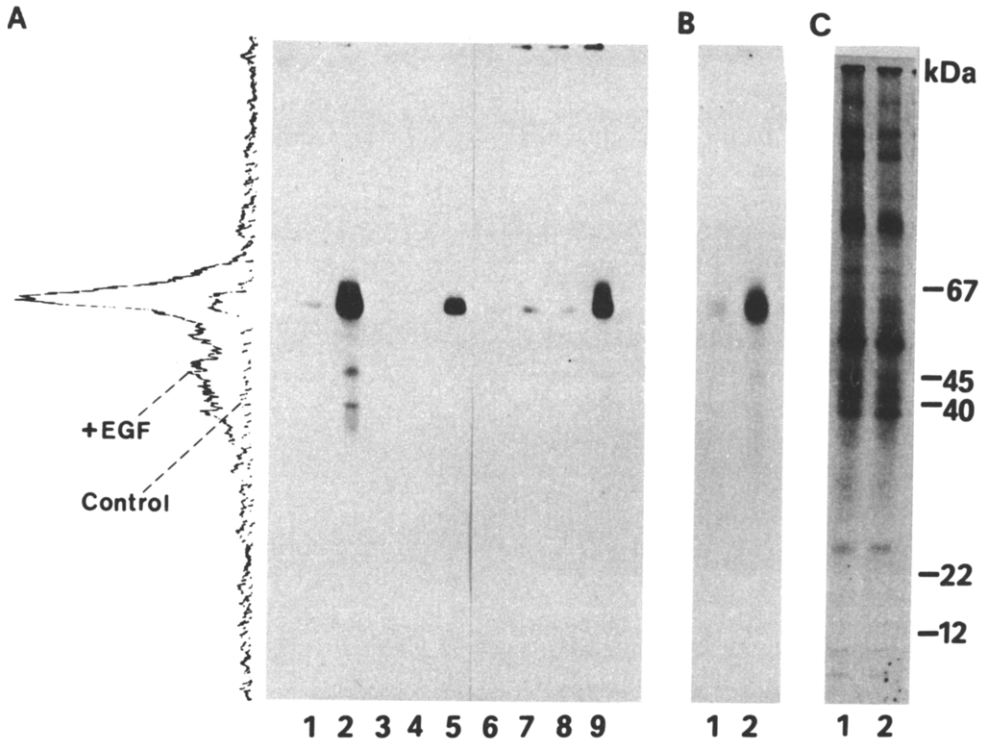

Fig.1. Effect of EGF and other growth factors on the pattern of phosphorylated proteins secreted by NRK cells. A, cells were labelled with $^{32}P_i$ for 3 h in the presence of control buffer (lane 1), 4 ng/ml EGF (lane 2); 4 ng/ml EGF and additionally either 20 μg/ml cycloheximide (lane 3), 20 mM NH_4Cl (lane 4), or 1 μg/ml actinomycin D (lane 5); in the presence of 4 ng/ml PDGF (lane 6), 10 ng/ml NGF (lane 7), 10 μg/ml insulin (lane 8) or 100 ng/ml TPA (lane 9). A radioactivity profile obtained by scanning the dried gel is also shown. B, in these experiments, the total radioactivity present during metabolic labelling was increased in order to enhance the basal ^{32}P incorporation into pp58; 5 ng/ml of TGF-β (lane 1), control buffer (lane 2). Autoradiographic exposure time: 16 h for both A and B. C, cells were labelled with [^{35}S]methionine (30 μCi/ml, 3 Ci/mmol) under the same conditions as in panel A. Lanes: 1, control; 2, 4 ng/ml EGF. Time of fluorography: 4 weeks.

The amount of radioactivity associated with pp58 was found to depend on the EGF concentration (fig.2). At concentrations of about 10^{-9}–10^{-10} M, a marked increase of pp58-associated radioactivity was observed. This concentration is similar to the K_d value determined for the binding of EGF to its receptor [17].

Next, the reversibility of the effect of EGF on the amount of $^{32}P_i$ incorporated into pp58 was examined (fig.3). When cells were transiently exposed to EGF, then incubated overnight without EGF in a fresh serum-containing medium and finally labelled with $^{32}P_i$ the radioactivity in pp58 (lane 5) was reduced to the basal level (lane 3). For control, it is shown that both under standard conditions (lanes 1,2) and after the overnight incubation cells responded to EGF (lane 4). Apparently, fresh medium changed the basal level of pp58 (cp. lanes 1,3).

The phosphorylated amino acid in pp58 was identified (fig.4). It is important to note that 60–70% of the total protein-bound radioactivity in the medium was associated with pp58. Indeed, the same pattern as that shown in fig.1 was found in gels dried and autoradiographed without prior fixation, staining and washing steps. Therefore, the culture medium was subjected to hydrolysis directly. The autoradiogram (fig.4) showed that only phosphoserine occurred in pp58. No phosphorylated sugar was found in the culture medium

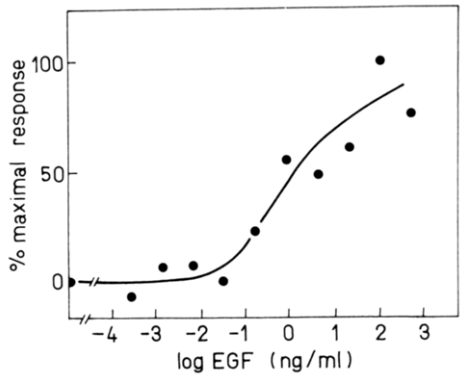

Fig.2. Effect of EGF concentration on incorporation of $^{32}P_i$ into pp58. Relative radioactivities were determined from the area corresponding to the position of pp58 after gel scanning.

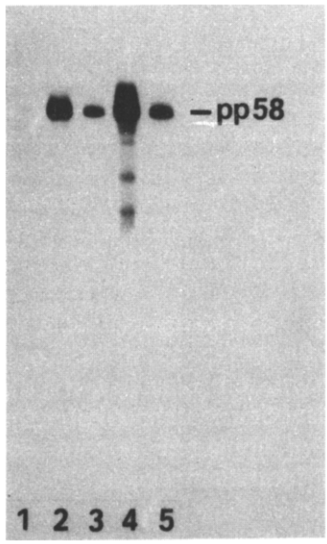

Fig.3. Demonstration of reversibility of EGF-dependent $^{32}P_i$ incorporation into pp58. NRK cells were incubated under standard conditions in five separate flasks (lanes 1–5) in the presence of 4 ng/ml EGF (lanes 2,5) or in the absence of EGF with the control buffer (lanes 1,3,4) for 3 h with (lanes 1,2) and without $^{32}P_i$ (lanes 3–5). After 3 h culture fluids from two of the flasks (lanes 1,2) were collected. The other flasks (lanes 3–5) received fresh medium with 10% calf serum and were cultured for another 19 h period. The medium was then changed for the serum- and phosphate-free medium and the cells were labelled for 3 h with $^{32}P_i$ in the presence (lane 4) or absence (lanes 3,5) of 4 ng/ml EGF. Normalized amounts of radiolabelled medium proteins were analysed as outlined in section 2.

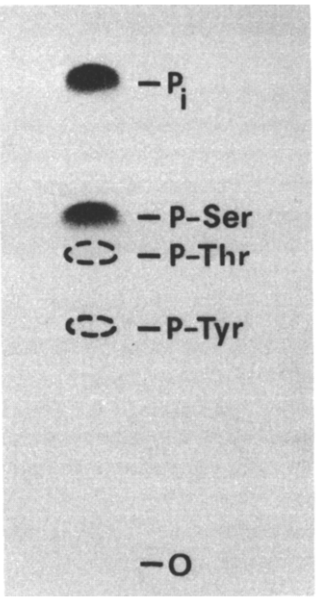

Fig.4. Identification of the amino acid phosphorylated in pp58. NRK cells were labelled for 3 h with $^{32}P_i$ in the presence of 4 ng/ml EGF. The secreted proteins were precipitated, washed, hydrolysed, and the hydrolysis products separated on cellulose plates for autoradiography. Phosphotyrosine (P-Tyr), phosphothreonine (P-Thr) and phosphoserine (P-Ser) were added as internal standards and detected with ninhydrin. No radioactivity was detected in the areas where P-Tyr and P-Thr migrated (dashed outlines). O, origin.

when analysed as described in [16]. This further distinguishes pp58 from secreted lysosomal hydrolases [16].

The mechanism whereby EGF increases the amount of radioactive phosphate in pp58 is not yet known. Upon labelling the cells with [^{35}S]methionine analysis of the secreted proteins did not reveal any increase in the relative abundance of pp58 in the presence of EGF (fig.1, panel C). This finding indicates that EGF may exert its effect by changing some phosphorylation step rather than by altering selectively the amount of protein which is constitutively phosphorylated.

ACKNOWLEDGEMENTS

The assistance of Mrs H. Kistel and E. Hellmuth is greatly acknowledged. We thank Mr L. Anders for help with the phosphoamino acid analysis and

Professor T. Rapoport for critical reading of the manuscript.

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